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14. ABSTRACT

The cytochrome P450 enzyme CYP1B1 is a major enzyme involved in metabolizing polycyclic aromatic hydrocarbons (PAHs) to reactive intermediates. Given that exposure to carcinogenic PAHs may be a breast cancer risk factor, we have set out to investigate molecular mechanisms of the relationship between PAH exposure, *CYP1B1* expression and breast cancer risk in a clinic-based case-control study.

We have collected nontumor breast tissue from 43 women (32 cases and 11 controls) undergoing surgery and analyzed these specimens for *CYP1B1* gene expression, CYP1B1 genotype and PAH-DNA adducts. CYP1B1 transcript levels determined by quantitative RT-PCR, varied more than 1000-fold between individuals. DNA adduct levels varied 10-fold between individuals.

Because of the delayed start of the project, we have applied for and obtained a no cost extension until 10/27/07.

15. SUBJECT TERMS
xenobiotic metabolism, CYP1B1, gene expression, genetic polymorphism, DNA adducts

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INTRODUCTION

This study investigates underlying molecular mechanisms of the relationship between PAH exposure and breast cancer risk. In breast tissue, the cytochrome P450 enzyme CYP1B1 appears to be a major enzyme involved in metabolizing PAHs to reactive intermediates (Goth-Goldstein et al., 2003). High CYP1B1 enzyme levels may result in increased formation of PAH-DNA adducts in breast tissue, subsequently leading to development of breast cancer (Lagueux et al., 1999). Gene expression analysis captures the convergence of multiple genetic and environmental factors that influence metabolic enzyme levels (Gonzalez and Gelboin, 1994; Whitlock, 1999). Using a clinic-based case-control design, breast tissue is being obtained from female patients undergoing either mastectomy or reduction mammoplasty surgery in Porto Alegre, Brazil. PAH exposure and potential confounding factor data are being collected for all cases and controls via medical chart review and an interviewer-administered questionnaire. To characterize molecular level inter-individual variation in PAH metabolism, *CYP1B1* gene expression and PAH-DNA adducts are measured in the surgically obtained breast epithelial cells from at least 37 cases and 74 controls undergoing mastectomy and reduction mammoplasty surgery, respectively.

BODY

Task 1: **Identify study participants undergoing reduction mammoplasties or mastectomies and collect data and tissue** – in progress

As mentioned in the last report, enrollment of participants has been slower than anticipated. For cases the reason is a recent change in standard of care that chemotherapy be administered before surgery for women undergoing a full mastectomy surgery. This conflicts with our eligibility criteria of no prior chemotherapy. Therefore, we are now collecting tissue from women undergoing partial mastectomy. Recruitment of controls has also been much slower than anticipated. Our Brazilian Co-Investigators have given two reasons: a change in beauty perception and the economic situation in Brazil makes the often elective surgery less affordable for many women.

At this time, we have recruited 43 women (32 cases, 11 controls) and obtained their consent to participate in the study. Urine samples were collected and breast tissue and blood samples obtained during scheduled surgeries. The collected specimens were processed by the clinical research coordinator and stored in a stabilizing buffer (RNA*Later*, Ambion) at -20^{o}C until shipment to LBNL. We have received the specimens of these participants in 3 shipments. So far, questionnaires were administered to 26 participants and 18 questionnaires have been hand-carried to the US and are awaiting data entry and analysis.

Table 1. Characteristics of participants recruited so far

	Total	Controls	Cases
Number	43	11	32
Age (mean)	52.3	43.7	55.2

Task 2: **Characterize the breast tissue samples in respect to *CYP1B1* expression and *CYP1B1* polymorphism** – in progress

a. Isolate DNA and RNA from breast epithelial cells

DNA was isolated by the Proteinase K - phenol/chloroform methods from the 43 specimens received. We had originally proposed to isolate the DNA together with RNA in the TRI Reagent procedure, but found that this method yielded only small amounts of low-quality DNA. DNA was quantitated and its purity determined by its 260/280 nm absorption. Samples were aliqoted for later measurements of CYP1B1 genotype and DNA adducts and stored at -80^{o}C.

Total RNA of the 43 specimens was isolated using TRI Reagent following the manufacturer's protocol (Molecular Research Center, Inc., Cincinnati, OH) and RNA samples stored at -80^{o}C.

b. Reverse transcribe RNA and

c. Measure *CYP1B1* gene expression

The *CYP1B1* expression level in the breast specimens is measured using real-time RT-PCR (with the LightCycler instrument). In order to accurately measure the quantity of gene transcript, each run of the LightCycler includes a dilution series of a CYP1B1 quantitation standard (QS). The standard and the CYP1B1 gene transcript are reverse transcribed and amplified together at equal efficiencies to control for each step of the assay. The CYP1B1 expression level in an unknown sample is determined by extrapolating from a curve produced from the dilution series of the quantitation standard. To control for variations in specimen quantities, an equal amount (500 ng) of total RNA isolated from each specimen is added to each RT-PCR reaction. Each reaction includes a negative control, a positive control (RNA isolated from HMEC 184 cell line) and a reverse transcription control.

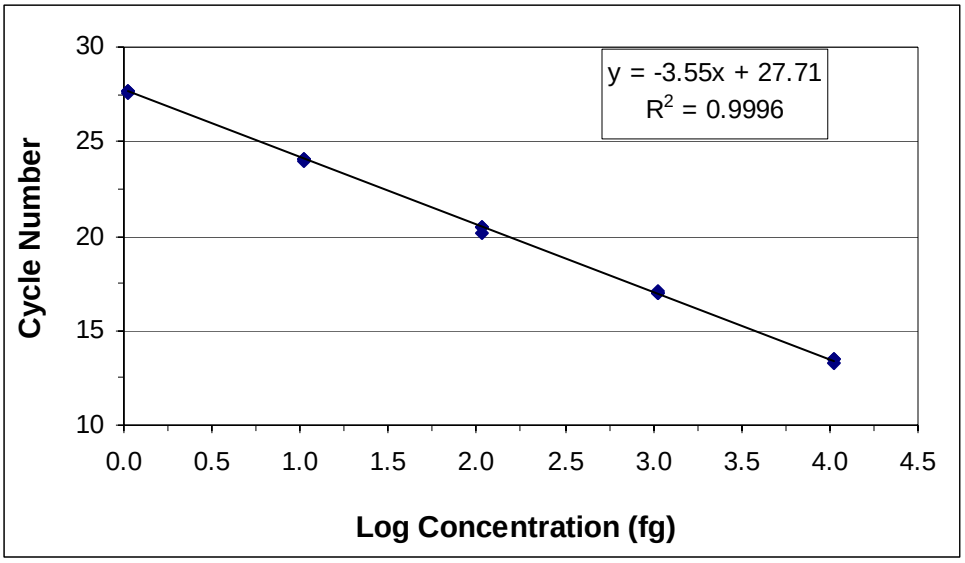

Figure 1. Triplicate analysis of the CYP1B1 Quantitation Standard (QS) using the LightCycler instrument. Each concentration level was measured three times (there are three data points plotted at each concentration level) with a variance of less than 1%.

Two possible sources of variation in the quantitative PCR assay were characterized, the reproducibility of the QS dilution series and the variability in the RNA isolation. The QS dilution series was tested in triplicate.

Figure 1 shows the analysis of the QS standard at five different concentration levels (from 10,000 fg to 1 fg), each in triplicate. The variance is less than 1% at each concentration level so that the data points in the figure appear overlaid.

To measure the variability in the isolation procedure, the RNA from one specimen (#128) was isolated in triplicate and then analyzed by the RT-PCR assay for CYP1B1. The average transcript level was 9.93 fg per 500 ng total RNA (SD = 0.66). The variance is 6.6 %; however, an initial measurement of the CYP1B1 transcript levels made from RNA isolated from specimen #128 six months earlier resulted in a higher level, 39.50 fg per 500 ng total RNA. This suggests that storage time of the specimen is critical and that RNA should be isolated as soon as possible.

Table 1. Results from CYP1B1 gene transcript measurement of specimen #128 with RNA isolation in triplicate. Transcript levels are expressed as fg/500ng total RNA used in the reverse transcription.

Specimen ID	CYP1B1 (fg/500ng RNA)
128-1	9.34
128-2	10.64
128-3	9.81

RNA*Later* turned out to be very effective in preserving the RNA in the specimens, so that even specimens from the first shipment, which arrived at room temperature, provided RNA and the quantitation of CYP1B1 transcript was comparable to repeat measurements with additional tissue sent in the second shipment. Of the 43 breast tissue specimens analyzed so far for their *CYP1B1* expression levels, 3 did not give any results. The values for the 40 specimens that gave results are summarized in Table 2. It shows that expression levels vary over a broad range.

Table 2. *CYP1B1* expression in 40 breast tissue specimens analyzed. Transcript levels are expressed as fg/500ng total RNA used in the reverse transcription.

	CYP1B1 Mean (SD)	range
Total (n = 40)	23.95 (19,66)	0.06 – 73.7
Control (n = 11)	17.27 (21.09)	0.078 – 54.5
Cases(n = 29)	26.48 ((19.78)	0.06 – 73.7

d. Perform *CYP1B1* genotype analysis

The *CYP1B1* genotype at two polymorphic sites located in the catalytic side of the enzyme at codon 432 (m1) and at codon 453 (m2) was analyzed by PCR /RFLP. Using the primers described by Bailey et al. (1998, corrections, 1999) a 144 bp product is amplified. This product can be used to detect both the m1 and m2 polymorphisms. The m1 (Val to Leu) polymorphism is detected by digestion with the restriction enzyme Eco571, which produces 83bp and 61bp fragments in the variant. The m2 (Asp to Ser) polymorphism is detected by digestion with Cac8I, which produces 106bp and 38bp fragments in the variant. The digestion products are separated on a 10% native polyacrylamide gel stained with SYBR Gold (Figure 2).

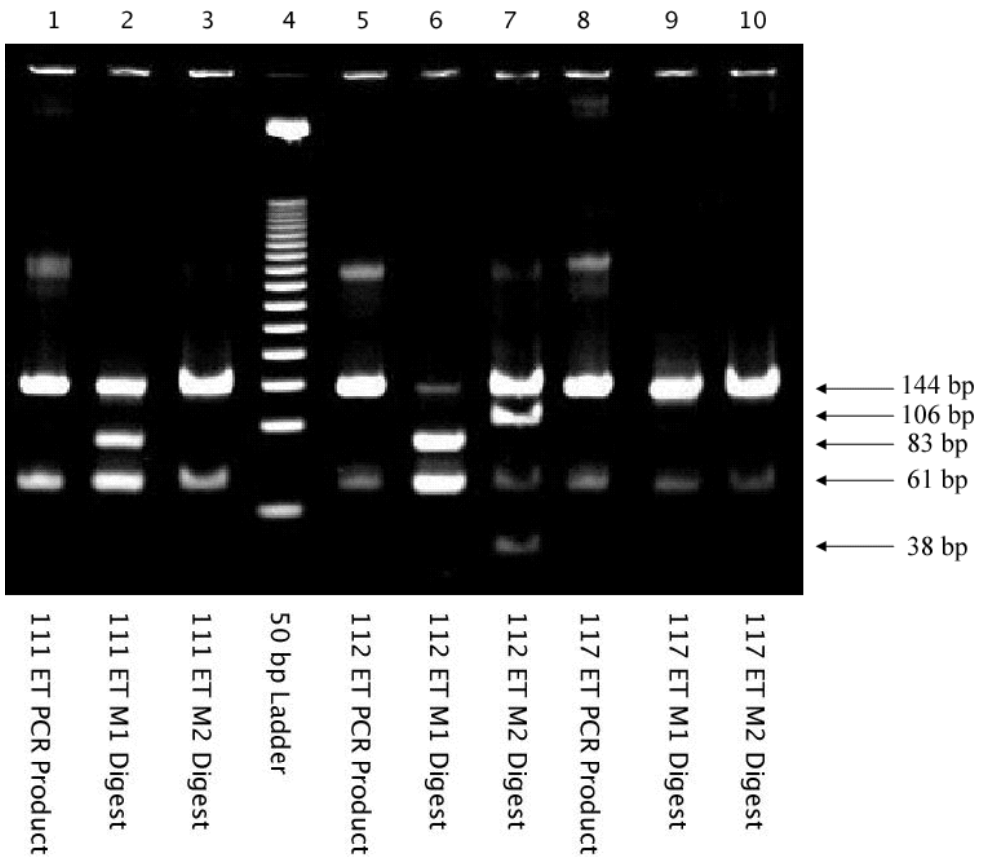

Figure 2: This gel shows the results for 3 specimens. Lane 1, 5, 8 contain the undigested PCR product (144 bp); lane 2, 6 and 9 contain the m1 digestion products (144bp, 83 bp and 61 bp); lane 3,7, 10 contain the m2 digestion products (144bp, 106 bp and 38 bp). Extraneous bands are visible at approximately 65 bp and 400 bp.

The results from the genotype analysis are summarized in Table 3.

Table 3. *CYP1B1* genotype of initial 43 participants.

CYP1B1 genotype		Total	Controls	Cases
m1	Val/Val	5	1	4
	Val/Leu	26	10	16
	Leu/Leu	12	0	12
m2	Asn/Asn	33	9	24
	Asn/Ser	9	2	7
	Ser/Ser	1	0	1

We calculated the allele frequencies for the two polymorphic sites and compared them to reports of allele frequencies observed in different ethnic groups. The allele frequencies determined in our small sample set resemble those seen in populations of European descent.

Table 4. Comparison of *CYP1B1* allele frequency in participants and in different ethnic groups (given as means of several published reports)

Codon	Participants	African Descent [1, 3]	Asian Descent [2, 3]	Europ. Descent [1, 2, 3]
432 Val	0.419	0.703	0.154	0.404
432 Leu	0.581	0.297	0.846	0.596
453Asn	0.872	0.975	0.997	0.807
453Ser	0.128	0.025	0.003	0.193

[1] Bailey et al.,1998; [2] Inoue et al., 2000; [3] Mammen et al.,2003.

Task 3 **Measure PAH-DNA adducts by ^{32}P-postlabeling in breast tissue** – in progress

Aliquots of DNA isolated from breast epithelial tissue are shipped to Dr. Donghui Li, a Co-Investigator at the M.D. Anderson Cancer Center, for DNA adduct analysis. Aromatic DNA adduct levels are determined by the nuclease P1-enhanced version of the ^{32}P-postlableing assay as described in Li *et al*. (1996), which involves stepwise DNA digestion to nucleosides, conversion to ^{32}P-labeled deoxyribonucleosides, purification and separation by multidirectional TLC. Adducts are detected and quantitated by image analysis. Adduct levels are expressed as a relative adduct level (RAL) value, which is a ratio of the counts per minute (cpm) of modified nucleotides over the cpm of total nucleotides in the reaction.

For 12 specimens, not enough DNA was obtained for adduct analysis. But even for the remaining DNA samples problems were encountered. Preserving tissue in RNA*Later*, a supersaturated salt buffer, appears to be a problem for DNA adduct measurements. We presume that carry-over of salts from the RNA*Later* inhibits nuclease digestion of the isolated DNA. We have modified the DNA isolation protocol to using a low-salt buffer and have reprecipitated the DNA to remove these salts but still several DNA samples did not give any DNA adduct results. We plan to use desalting columns to remove extraneous salt.

Table 5. PAH-DNA adducts determined in initial specimens (expressed as RAL x 10^9)

	Mean (SD)	range
Total (n = 20)	32.00 (20.36)	0 – 93.06
Controls (n = 3)	29.23 (17.10	16.19 – 48.59
Cases (n = 17)	32.49 (21.3)	0 – 93.06

Task 4 **Analyze data and write reports** have not been initiated because of the delay in recruitment. We have requested and obtained a no-cost extension until 10/27/07.

KEY RESEARCH ACCOMPLISHMENTS

- Enrolled 43 participants and brought collected specimens in three shipments to LBNL
- Isolated DNA and RNA from all epithelial breast tissue specimens
- Determined *CYP1B1* expression in all specimens, got results for 40 of 43
- Determined *CYP1B1* polymorphism at codon 432 and codon 453 in all specimens
- Determined DNA adducts, so far only results for 20 out of 43

REPORTABLE OUTCOMES

None

CONCLUSIONS

After long delays, we have now analyzed the first 43 breast tissue specimens for *CYP1B1* gene expression, *CYP1B1* polymorphism and PAH-DNA adduct level to test our hypothesis that high *CYP1B1*expression results in high levels of PAH-DNA adducts and increases the risk of breast cancer. Since we have recruited only a third of the planned participants and analyzed their samples, we cannot evaluate the validity of our hypothesis yet. However, we have observed a wide range of values both for CYP1B1 transcript levels and for PAH-DNA adducts.

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